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Role of Ubiquitin E3 Ligase Breast Cancer Associated Gene 2 (BCA2) in SUMOylation

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Thesis

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Abstract

As the occurrence of breast cancer increases around the world, the need for new therapeutic medicine becomes more vital to sustain the population. In order to accomplish this, specific mechanisms need to be understood to gain a perspective on the overall process of breast cancer development. One mechanism that is being researched is SUMOylation. The SUMOylation reaction uses small ubiquitin-like modifier (SUMO) proteins, which act as a type of post-translational modification that is necessary in order to preserve protein homeostasis and regulate cellular processes including apoptosis, cell proliferation, and response to stress. The SUMOs attach to a target protein through a SUMO E3 ligase and the modified protein then gains different cellular functions. An example of the SUMO-mediated protein degradation involves Arkadia, a SUMO-targeted ubiquitin ligase, which stimulates the degradation of the Ski protein, which is associated with tumor growth and development.

Breast Cancer Associated Gene 2 or BCA2 is identified as an E3 ligase in the ubiquitin proteasome system. It has been found that BCA2 is co-expressed with the estrogen receptor (ER) in ER-positive breast cancer cells. ER is crucial in the regulation of cell cycle progression and growth of breast cancer epithelial cells. Previous studies have shown that the BCA2 gene is regulated by the ER and BCA2 protein interacts with UBC9 and E2 ligase in the SUMOylation pathway. The regulation of BCA2 could prove critical since it could mediate the ubiquitination and possible SUMOylation of target proteins. While BCA2 as an Ubiquitin E3 ligase has been well established, its role in SUMOylation is unknown. The aim of this thesis is to identify BCA2's involvement in SUMOylation. To achieve this, I performed bioinformatics analysis of the BCA2 protein sequence and found that out of the 6 lysines (Ks) in the sequence, K32 is the possible lysine important for SUMO attachment. I also tested the SUMOylation of BCA2 in an

in vitro assay by using SUMO-specific proteins. The results have revealed an association between SUMO 2/3 and BCA2. Plasmids containing SUMO 1 and 2/3 were then purified from Top10 bacterial cultures and were transfected into HEK293 cells for immunoprecipitation experiments. Preliminary data from these experiments reveal a possible interaction of BCA2 with SUMO2/3. The involvement of BCA2 in both ubiquitination and SUMOylation pathways as an E3 ligase makes it a novel target for therapeutic intervention for the prevention and treatment of breast cancer patients.

Introduction

Cancer is a disease that has become increasingly prevalent in the world population in the recent years and is predicted to continue to be the second most common cause of death in the United States. In fact, according to the American Cancer Society, 1,660,290 new cancer cases are expected to be identified, while 580,350 Americans are expected to die of cancer in this year alone [1]. Worldwide, it is predicted that by 2020, the world population will be 7.5 billion and 15 million new cancer cases will be diagnosed [2]. Because of this constant increasing predominance, it is vital to learn how cancer cells function in the human body in order to develop new and more effective treatment options. This life-changing disease results from an uncontrolled growth of transformed human cells, which are thus called cancerous cells. Under normal conditions, cells are regulated with immense precision and accuracy by signals released by the body's tissues. When cells have served their purpose or have become old, the body efficiently removes them from the system either by apoptosis or by phagocytic cells in a process called efferocytosis [3]. When a tissue is damaged, normal cells grow until the tissue is completely healed. However, in cancer, these seemingly normal human cells continue to divide

at an undisciplined rate until a tumor is formed that could then metastasize and move to other organ systems to spread the disease. Though many causes of cancer may be uncontrollable, it is also caused by lifestyle preferences that people could change, including smoking, diet, exercise, and obesity, in addition to others [4].

Since each tissue and organ in the human body is composed of cells, essentially every part of the body is susceptible to cancer. As a result, many forms of cancer arise with each organ that is affected. One type of cancer that is becoming progressively more widespread is breast cancer. According to U.S. National Library of Medicine, 1 in 8 women will be diagnosed with this type of cancer during their lifetime [5]. Additionally, the American Cancer Society states that breast cancer is the most common cancer among women in the United States, excluding skin cancer [6]. For this reason, it is crucial to continue research in order to facilitate the discovery of new drugs and treatment options.

One strategy for improving breast cancer treatment is to explore the mechanisms of protein molecules that affect growth and proliferation of breast cancer cells and their regulations. Cellular homeostasis is necessary in order to maintain cell function and regulation. For this reason, many of the cellular processes that are vital to a cell, such as apoptosis and transcription, are highly controlled with specific proteins preserving the different mechanisms and occurrences of a functional cell. These proteins and molecules are, themselves, kept in check by various other molecules so that no single molecule has complete control of the cell and its interactions. This allows the numerous proteins to work in conjunction with one another in order to keep the cell check-and-balanced and its proliferation rate regulated so that cells do not continue dividing ungoverned and do not become cancerous within the body's tissues.

One such vital regulatory protein that is found in most eukaryotic organisms is ubiquitin [7]. This highly conserved protein can attach to various proteins and direct them to the proteasome for degradation in an enzymatic process called ubiquitination [8]. Since this protein decides which proteins are still needed by the cell and which proteins can be discarded, ubiquitin is necessary to maintain the customary functions of a eukaryotic cell and a defect in this process can cause many diseases, including cancer [9]. In fact, ubiquitin is essential in protein modification and protein turnover, as well as the balance between synthesis and degradation of proteins, making this protein a necessity for functional cells [10, 11,12]. Ubiquitin marks a protein for degradation with three steps. First, the protein is activated by an ubiquitin-activating enzyme (E1); next the ubiquitin is transferred from the E1 to an ubiquitin-conjugating enzyme (E2); finally, the conjugating E2 enzyme is added to the target protein by an ubiquitin ligase (E3) [13]. The E3 ubiquitin ligases are known to play a role in cell proliferation, apoptosis, and cancer invasion [13]. Ubiquitin E3 ligases allow substrates to bind to the ubiquitin-proteasome complex for degradation. There are two domains an E3 ligase can contain: a HECT domain and a RING domain [14]. Therefore, E3 ligases are found to be a therapeutic target since its deregulation is known to be a part of the proliferative diseases [15].

An E3 ligase with a RING domain is Breast Cancer Associated Gene 2 or BCA2 [16]. This protein, which was identified by hybridization cloning of cDNA using normal and cancerous breast cells [17], is known to be expressed in breast cancer cell lines [16]. In addition to a RING domain which is vital for its autoubiquitination, BCA2 also has a zinc finger domain.

BCA2 is expressed in primary invasive breast cancers and is associated with a positive estrogen receptor status [15, 18]. As shown in Table 1 of ref. [17], BCA2 is expressed in 74% of ER-positive invasive breast cancers. Estrogen regulates the proliferation and development of

tissues that express ER and therefore, estrogen and ER can be a risk factor for breast cancer development [16]. It has been shown that BCA2 is a target of ER transcriptional activity in ER-positive breast cancer cells and that the BCA2 gene is up-regulated with more of the hormone [19].

It has been proposed that BCA2 may be involved in the attachment of SUMO proteins [16]. SUMOylation is a type of post-translational modification that is involved in many cellular processes including transcriptional regulation, apoptosis, protein stability, and cell cycle progression [20]. The SUMOylation reaction uses small ubiquitin-related modifier (SUMO) proteins, which bind to the substrate protein with the help of E3 ligases, targeting them for either degradation or other cellular functions [16]. ER has SUMO-attachment sites and is activated when it undergoes SUMOylation [21]. Since ER is highly expressed in breast cancer patients, along with BCA2, SUMOylation of BCA2 needs to be studied further in order to better understand its role in breast cancer and if it can be a potential target for treatment options.

Furthermore, it has been shown that RING-E3 ligases are important in the SUMO pathway, since they stabilize proteins and compete for ubiquitin modification sites [22]. As a result, BCA2, as a RING-E3 ligase, interacts with Ubc9, an E2 enzyme in the SUMO pathway [16], which was discovered from pull down and immunoflourescence assays [17]. Additionally, it was observed that co-localization occurs between these two proteins in both the nucleus and the cytoplasm [17]. Therefore, BCA2 could act as an E3 component of the SUMOylation process and the regulation of this protein could prove a pivotal target for treatment options. In order to fully understand the involvement of BCA2 in the SUMOylation process, I have studied SUMOylation of BCA2 *in vitro* using SUMO- proteins and researched the role of BCA2 in SUMOylation.

Materials and Methods

In order to conduct experiments regarding SUMOylation of BCA2, the following materials and methods were used.

Cell Lines

Cells were originally purchased from the American Type Culture Collection. They include the BCA2-expressing breast cancer cell MCF-7 and HEK293. The cell lines were cultured in RPMI 1640 or DMEM media, which was supplemented with 10% Fetal Bovine Serum (FBS) and grown under standard conditions at 37ºC and 5% CO_2.

Recombinant BCA2

Human BCA2 encoding DNA was cloned into the pET100 expression vector (Invitrogen) and used in *in vitro* SUMOylation experiments. An expression vector is used to introduce a gene to a specific target in order for the target cell to stimulate protein synthesis of the protein encoded by the gene. The recombinant protein was purified by affinity chromatography using the ProBand Purification System from Invitrogen.

Sumoylation Assay and **Western Blotting**

Recombinant BCA2 produced from *E.Coli* was used for SUMOylation experiments by adding ATP (Sigma-Aldrich), Sumo 1 or 2/3 (Sigma-Aldrich), E1, and E2/Ubc9 in Tris•HCl, pH 8.0. The reagents were mixed with 10X reaction buffer and water for an incubation period of two hours at 30ºC under constant shaking in a shaking incubator running at 250 rpm. The reactions were separated on a SDS-PAGE Gel and then transferred to a membrane, where it was subjected to immunoblotting with anti-Sumo 1 and 2/3 antibodies. Immunoblotting is a technique that uses antibodies to identify the target protein through antibody-antigen interactions.

Also, the proteins from treated cells were then separated on 4-20% SDS-PAGE Gels (Invitrogen) and transferred to PVDF/nitrocellulose membranes. Primary antibodies were diluted in blocking buffer and incubated for one hour at room temperature. The membranes were then incubated with species-specific secondary antibodies conjugated to horseradish peroxidase for one hour. Signals were developed using a chemiluminescence kit (Millipore) and detected using a FOTODYNE Gel Doc system.

Immunoflourescence

Cells were added in 8-well chamber slides (30,000/well) and were allowed to grow to a maximal amount of 80% and then rinsed with Phosphate-Buffered Saline (PBS) and fixed with an ice cold methanol:acetone (1:1) mixture and air dried for fifteen minutes. Slides were then re-hydrated in PBS, followed by a one hour blocking of unspecific binding sites in 5% Bovine Serum Albumin (BSA)/PBS. For dual labeling, slides were incubated for two hours with a mixture of polyclonal and monoclonal antibody and developed with species-specific FITC/TRITC secondary antibodies. FITC/TRITC are isothicyanates that are used for protein fluorescent labeling. Nuclei were counterstained with DAPI, a fluorescent stain, for three minutes.

Plasmid Preparation

Transforming Plasmid DNA

Transformation is a way in which cells take up DNA molecules, and it can be replicated along with the cell's own DNA. This involves competent cells that can uptake DNA from their surroundings.

Antibiotic Selection

Not all of the competent cells will uptake the DNA; if they do, it does not necessarily mean the cells will transfer the DNA to its descendent cells. Therefore, in order to find out which cells contain the DNA plasmid, antibiotic selection is applied. This process includes growing the cells on antibiotic resistant agar plates, such as kanamycin or ampicillin. Cells with successfully transformed plasmids will be resistant to the antibiotic and be able to grow on the plate. These cells will form colonies, a visible cluster of growth on the plate originating from a singular cell that has procreated numerous times while in the incubation period.

Growth of single colony in Lysogeny Broth (LB) Broth

Among the colonies that have successfully grown, one is picked for further study. This colony is placed in LB Broth medium along with the antibiotic and allowed to grow overnight on a shaking incubator.

Cell Pellets

The single colony grown in LB broth is then isolated from the medium and the cells pelleted using centrifugation. Since the collection of cells is more dense then the medium, the cells collect in a white group at the bottom of the tube with the broth on top.

Plasmid Purification

Plasmid purification is a way to isolate and purify plasmid DNA. This preparation method depends on the size of the plasmid DNA. The one I used was Minipreparation, which is for small-scale plasmid DNA. This process included adding many buffers in order to wash the DNA and obtain the maximum yield.

NanoDrop

After purifying the plasmid, the DNA concentration is measured using the NanoDrop machine. This measures the quality and concentration of the DNA in the sample so that it can be compared to different strains and be used for further experiments.

Agarose Gel Electrophoresis

After obtaining the DNA concentration, an agarose gel is run in order to visually see the DNA and determine its weight in comparison to a DNA ladder, which is a solution of DNA molecules of varying lengths used as a reference to get an estimation of the size of the unknown DNA molecules from the sample cells. The one I used had the length of 1 kb. After making the gel and applying the various samples into the wells, a current is applied which will move the DNA fragments. Since DNA is negatively charged, it will migrate towards the positive electrode, colored red. By adding ethidium bromide, a fluorescent dye used for staining nucleic acids, the distance DNA has migrated can be seen. This dye intercalates between the bases of DNA. After the samples have run the length of the gel, the gel is placed under fluorescent light and the DNA is seen as dark bands against a gray background.

Immunoprecipitation

HEK-293 cells in 100-mm dishes were transiently transfected with a total of 5 μg of plasmid DNA (the blank vector pcDNA3 was used as filler DNA in single transfections). After 48 hours, the cells were washed with cold PBS and lysed in RIPA buffer (2.5 M NaCl, 0.5 M Tris pH 7.4, 0.5 M EDTA, 10% v/v Triton X-100, 1 mM PMSF and 0.2 mM Na_3VO_4) at 4 °C. Cell lysates were pre-cleared with Protein G beads, and were incubated with 1 μg of BCA2 (rabbit) antibody for 21 hours. After incubation, the beads were collected by centrifugation,

washed with cold RIPA buffer and re-suspended in sample buffer. For Western blotting of immunoprecipitates, SUMO 2/3, UBC9 and BCA2 (Goat) was used as primary antibodies at 1:1000 dilution. HRP-conjugated anti-Goat IgG or anti-mouse IgG antibodies were used as secondary antibodies at 1: 30,000. Blots were visualized using ECL Plus (GE Healthcare) on a FotoDyne.

Results

In order to predict the SUMO binding position on the BCA2 protein sequence, SUMOplot analysis program was used and the results of this analysis are shown in Figure 1. Based on this analysis, Lys32 is the possible SUMO binding site for the BCA2 protein which scored 0.5 while the remaining 5 lysines scored negative values (data not shown), suggesting only K32 could undergo SUMO ligation.

Figure 1: Predicted SUMO binding sites for BCA2

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AEASAAGAD SGAAVAAHRF FCHFCKGEVS PKLPEYICPR CESGFIEEVT
DSSFLGGGG SRIDNTTTTH FAELWGHLDH TMFFQDFRPF LSSSPLDQDN
NERGHQTH TDFWGARPPR LPLGRRYRSR GSSRPDRSPA IEGILQHIFA
FFANSAIPG SPHPFSWSGM LHSNPGDYAW GQTGLDAIVT QLLGQLENTG
PADKEKIT SLPTVTVTQE QVDMGLECPV CKEDYTVEEE VRQLPCNHFF
SSCIVPWLE LHDTCPVCRK SLNGEDSTRQ SQSTEASASN RFSNDSQLHD
RWTF
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No.	Pos.	Group	Score
1	K32	KGEVS **PKLP** EYICP	0.50

Figure 1: This shows a predicted binding site of BCA2 for SUMO proteins.

In order to study the role of BCA2 in the SUMOylation process, SUMOylation of BCA2 *in vitro* using SUMO-proteins was done using SUMO E1 and Ubc9 (Figure 2). As shown in Figure 2, a high molecular band was detected when WT BCA2 and a His- and GST-tagged SUMO 2/3 protein was used. Both of these tags were used to purify the SUMO proteins. Also, no bands were seen when RING BCA2 (mutant form of BCA2 which is ligase dead) was used with Sumo2/3 with either His Tag or GST Tag. Therefore, this data shows that SUMO 2/3 might be involved in the SUMOylation of WT BCA2. SUMO 1 was also tested and no high molecular weight bands were found (data not shown), further indicating that BCA2 might be associated with only SUMO 2/3.

Figure 2: *In Vitro* Sumoylation of BCA2 using Sumo E1 and Ubc9

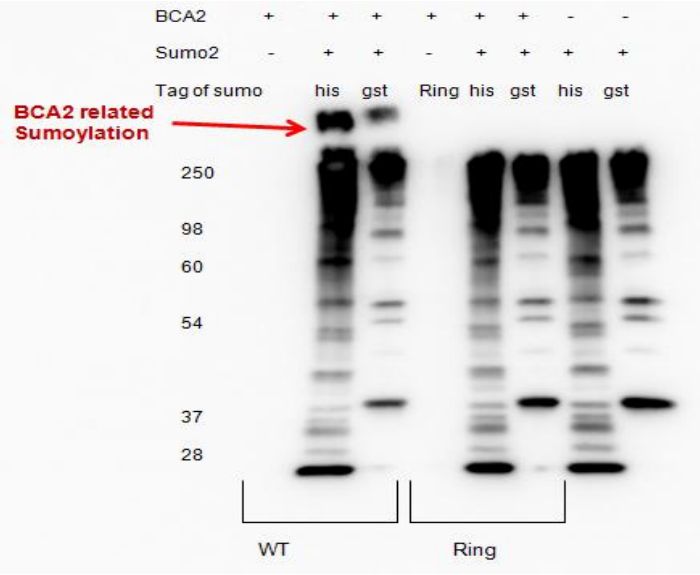

Figure 2: WT BCA2 and the RING mutants were tested for SUMOylation *in vitro* using SUMO E1 and Ubc9 in the presence of ATP.

After assessing that BCA2 and SUMO 2/3 might interact with each other, immunoflourescence experiments were carried out in order to test if these proteins co-localize with each other within the cells. Immunoflourescence staining of BCA2 in MCF7 cells was

performed and these images were then captured using a Leica DM5500 microscope and Improvision software with a Retiga camera (Figure 3). 4', 6-diamidino-2-phenylindole (DAPI) was used in order to stain nuclei of the fixed cells. BCA2 was stained red and SUMO 2/3 stained green. All three stains were then merged in order to see overlap. As can be seen from the enlarged Figure 3, the blue arrows indicate overlap of BCA2 and SUMO 2/3. This data shows the co-localization of BCA2 and SUMO 2/3 in the nucleus of MCF7 cells, suggesting a possible interaction found using *in vitro* sumoylation (Figure 2).

Figure 3: Co-localization of BCA2 and SUMO2/3 in the nucleus.

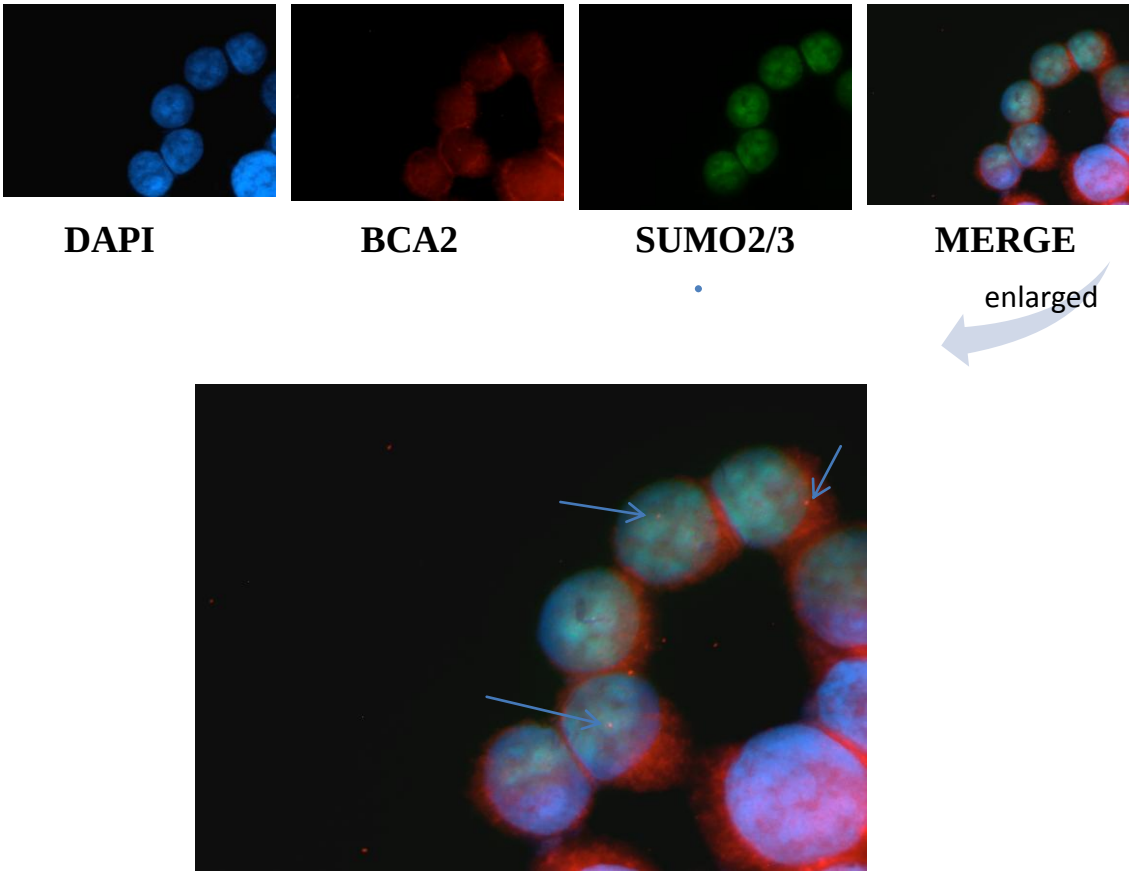

Figure 3: MCF7 cells immunoflourescence staining. BCA2 is stained red and SUMO 2/3 green. Co-localization of BCA2 (red) and SUMO 2/3 (green) in the nuclei (merge, blue arrows indicate the exact places of co-localization).

In order to identify the possible interaction of BCA2 and SUMO2/3, we purified the plasmid DNA from the bacterial cell cultures. These plasmids were a generous gift from Dr. Xiang Dong Zhang. After purification, the DNA of each colony was run on an agarose gel, (Figure 4).

Figure 4: Plasmid Purification Gel

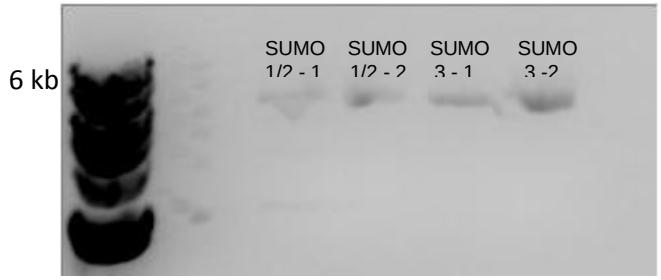

Figure 4: Agarose Gel of SUMO 1/2 and 3, colonies 1 and 2.

These plasmids were then used for transfection into HEK293T cells and for immunoprecipitation experiments. Immunoprecipitation is a technique where a protein antigen is precipitated out of solution using an antibody that binds specifically to that antigen. The antibody used was BCA2 (rabbit) and for the Western blot SUMO 2/3, BCA2 (Goat), and Ubc9 were used. As can be seen from Figure 5, the initial input contained a large amount of SUMO 2/3; when it was pulled down with BCA2, a clear signal was given in the Western blot, indicating that there is an association between BCA2 and SUMO 2/3. When this IP was probed for the Ubc9 signal, is strong signal showed up in the input as well as with pulldown with BCA2, which proves the previously established association between BCA2 and UBC9. Even though this is preliminary

data, further confirmation of association between BCA2 and SUMO2/3 interaction should be validated using IgG controls for immunoprecipitation experiments.

Figure 5: Immunoprecipitation of BCA2

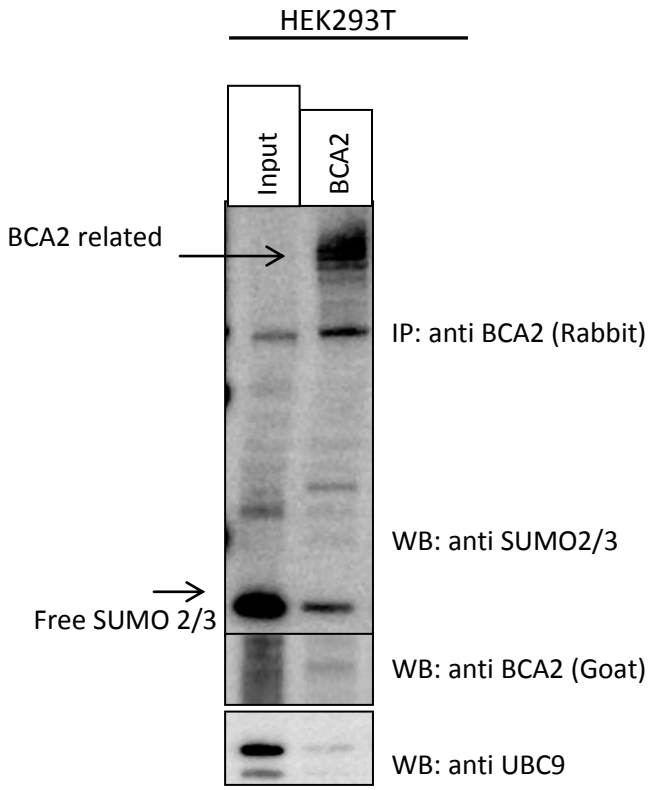

Figure 5: This shows the immunoprecipitation of BCA2.

Discussion

In order to study the role of BCA2 in the SUMOylation process, SUMO binding sites were predicted. From these bioinformatics analysis, K32 of BCA2 is the possible SUMO binding site. SUMOylation of BCA2 *in vitro* using SUMO-specific proteins was done using SUMO E1

and Ubc9. By using His Tag and GST tagged SUMO2/3 with WT BCA2 and RING BCA2, a possible SUMOylation between BCA2 and SUMO 2/3 were found. The dark bands, associated with BCA2 related SUMOylation, were only visible with WT BCA2 and not with RING BCA2. High molecular weight SUMO bands were seen for wild type BCA2 but not RING BCA2. This suggests that there is a correlation between BCA2 and SUMO 2 and 3, and this correlation is only present with SUMO 2/3 specific antibodies with the wild type BCA2 and not RING BCA2.

Furthermore, in order to detect possible protein-protein association, immunoflourescence staining of MCF7 cells were performed. This type of staining colors the areas the proteins are most prevalent in within the cells. BCA2 was stained red and SUMO 2/3 was green. By merging the three images (Figure 3) areas of overlap appear which designate places where an association between BCA2 and SUMO 2/3 is likely. It is clear that this overlap occurs within the nuclei of MCF7 cells, as indicated by the blue arrows. The co-localization of BCA2 and SUMO 2/3 show a possible interaction between them.

After preparing and purifying the plasmids, I ran an agarose gel in order to detect the amount of DNA in my samples of SUMO1 and 2/3. These plasmids were used for further transfection experiments.

Further experimentation of BCA2 and SUMO 2/3 performed was immunoprecipitation using the BCA2 antibody from rabbit to probe for SUMO 2/3, Ubc9 and BCA2 from goat. The Western blot experiment showed that a large amount of free SUMO 2/3 is seen in the input and there is a strong signal when it is pulled down with BCA2. This further confirms what was found in *in vitro* SUMOylation of BCA2 (Figure 2) and immunoflourescence imaging of MCF7 cells (Figure 3): there is an association between BCA2 and SUMO 2/3. Furthermore, the

immunoprecipitation data supports the findings of previous studies, which have established an association between BCA2 and Ubc9. Since there was a strong signal for Ubc9 in the Western blot after pulldown from BCA2, this indicates that BCA2 does interact with Ubc9 in the SUMOylation process and this is specifically related to SUMO 2/3. These results are very preliminary and with further studies and trials, better results are possible.

Conclusion

With the increasing prevalence of cancer, especially breast cancer, treatment is vitally important to stop the continuous growth of the cancerous tumors. However, in order to fully treat a patient, a complete understanding of the cancer is needed to effectively target the correct proteins or molecules that are part of the mechanism. For this reason, it is quite a feat to create a drug that accomplishes this since the human body is so diverse and complex with each small interaction having monumental effects.

One aspect for understanding tumor growth is SUMOylation, which uses SUMO proteins in order to preserve protein homeostasis. SUMO proteins could interact with an E3 ligase which helps them attach to the target protein for modification. BCA2 is an E3 ligase and is found to be co-expressed with estrogen receptors (ER). Estrogen, the primary hormone in women, is necessary for breast tissue development and regulates the proliferation of tissues that express ER. ER has SUMO-attachment sites and it undergoes SUMOylation when activated. Therefore, this study was conducted in order to reveal the association between BCA2 and SUMOylation of target proteins involved in breast cancer.

In order to find that association, *in vitro* SUMOylation of BCA2 was performed using Ubc9, a known E2 ligase, and SUMO E1, as well as immunoflourescence staining, plasmid purification, and immunoprecipitation. From these results it was revealed that BCA2 might interact with SUMO 2 and 3 in the SUMOylation pathway. Since BCA2 is highly expressed in breast cancers, its regulation and degradation could prove to be a vital aspect in finding new treatment options and drugs for battling breast cancer.

Even though an association between BCA2 and SUMO 2 and 3 has been found through this study, further experimentation in this mechanism will be needed in order to fully understand the implications behind this and other processes that are closely linked with breast cancer in order to improve the current cancer prevention and treatment strategies.

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